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13. ABSTRACT ***(Maximum 200 Words)***

The goal of this research has been to develop an immune-based gene therapy that combines targeted cytotoxicity with reversal of local tumor immune suppression to eradicate prostate cancer cells. Reversal of local tumor immune suppression was achieved *in vitro* by blocking the over expression of TGF-β2 produced by prostate cancer cells using TGF-β2 phosphorothioate oligonucleotide antisense. We have been developing a gutless adenovector with extended transgene expression that we think will have enhanced safety for pre-clinical and clinical use. Our gutless adenovector DNA backbone contains the cytotoxic gene under the control of an improved prostate specific promoter and a marker GFP gene for detection in *in vivo* studies. Our improved PSA promoter/enhancer presents 19-fold higher transcriptional activity compared to native PSA promoter/enhancer and has no loss of tissue specificity. Using Apo2L/TRAIL, a TNF-α related cytokine with less systemic toxicity, we have demonstrated selective cytotoxicity in our highly aggressive androgen-independent prostate cancer cell line (CL1). Further *in vivo* studies are being conducted to evaluate the overall efficacy and safety of Apo2L/TRAIL gene therapy in combination with TGF-β2 antisense for prostate cancer treatment.

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Introduction:

Gene therapy can selectively introduce therapeutic genes into cancer cells. However, a variety of vector properties must be improved to assure efficient and targeted anti-cancer gene delivery. Our laboratory has been developing an improved gutless adenovector to achieve not only appropriate levels of therapeutic gene expression in cancer cells but also to decrease vector immunogenicity. Our gutless adenovector is devoid of all viral coding sequences except for terminal sequences required for viral replication and packaging, and is propagated using a helper-dependent system that provides all the necessary viral gene products in trans using 293Cre 4 cells (1,2). Using our gutless adenovector as a therapeutic gene delivery system, we expect to eliminate interference with the activity of the PSA promoter/enhancer sequences (3-5) and minimize cellular and humoral immune responses elicited by viral proteins (6,7).

The transcriptional activity of tissue-specific promoters, including PSA promoter/enhancer, is much lower than constitutive viral promoters such as CMV. To attempt to improve the safety and efficacy of gene therapy *in vivo*, we developed a PSA promoter/enhancer with enhanced activity and good tissue-specificity. Based on principles of transcription regulation, we manipulated known PSA regulatory components by insertion and duplication of high affinity androgen-responsive elements (AREs) and by removal of intervening sequences between the enhancer and promoter sequences. Our *in vitro* studies demonstrate that our PSA-BC promoter/enhancer construct not only exhibit 19-fold higher activity relative to the PSA promoter/enhancer control baseline, but also retained a high degree of tissue-specificity.

To achieve maximal targeted immune stimulation in immune-triggering gene therapy, intra-tumoral immune suppression must be reversed. TGF-β is over expressed in high grade prostate tumors and correlates with immune suppression, disease progression, recurrence rate, therapeutic resistance, and poor prognosis (8-11). Reversal of intratumoral immune suppression by down regulation of TGF-β may facilitate the efficacy of immune-based TNF-α gene therapy. In our studies we used antisense against TGF-β2, rather than TGF-β1, because of higher immune suppressive effects on PBL activity. *In vitro* down-regulation of TGF-β2 by TGF-β2 phosphorothioate oligonucleotide antisense inhibited prostate cancer cell growth and decreased immune suppressive effects on PBL proliferation.

TNF-α is one of the most potent cytotoxic cytokines, with anti-tumor effects including direct cytotoxicity, induction of hemorrhagic necrosis, and immune modulation activity (12-14). We evaluated the cytotoxic effects of Apo2L/TRAIL compared to TNF-α in prostate cancer cell lines. Apo2L/TRAIL, in much lower concentrations than TNF-α, demonstrated enhanced tumor-selective mediated cytotoxicity with less systemic toxicity. Further *in vivo* studies are being conducted to evaluate the efficacy and safety of Apo2L/TRAIL gene therapy using CMV and improved PSA promoter/enhancer.

Body:

Task 1. *Development of a gutless adenovector for gene therapy targeting gene expression under the control of PSA promoter/enhancer in order to improve safety and specificity of prostate cancer gene therapy.*

We initially encountered major problems with recombination in generating our gutless adenovector. Recombination occurred between our gutless adenovector DNA backbone and the helper virus DNA during the serial passages of the vector in cells co-infected with helper virus. Such homologous recombinations during amplification passages have been reported (15). To decrease vector rearrangement during serial passages of the vector in 293Cre4 cells, we changed the stuffer to a different 25 Kb human DNA fragment (HPRT + C346 cosmid sequences)

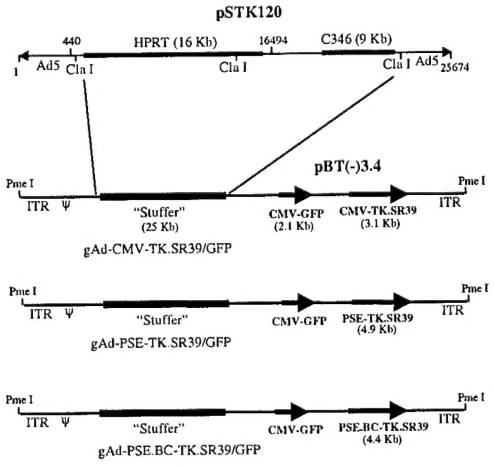

Figure 1. Generation of "gutless" Ad constructs containing HSV TK.SR39 and marker GFP. The ITR sequences and the packaging signal ψ are flanked by unique PmeI sites that will be used to release the vector fragment from the pBT3.4 plasmid backbone before transfection into 293Cre4 cells for viral production and propagation. The 25 kb stuffer was obtained from pSTK120 plasmid. The directions of therapeutic transgenes (CMV-TK.SR39, PSE-TK.SR39, and PSE.BC-SR39) and marker gene CMV-GFP are indicated.

which increased the gutless adenoviral DNA backbone to 30-32 Kb, critical for optimal packaging efficiency (Figure 1). The choice of DNA stuffer sequence has been shown to affect vector replication and stability during propagation (16,17). These new vectors are being amplified and will be tested for DNA integrity and purity prior to *in vivo* studies. To evaluate our improved PSA promoter we are using Herpes Simplex Virus Thymidine Kinase/SR39 (HSV-TK.SR39, a more active variant of HSV-TK) to trace the gutless adenovector *in vivo* with non-invasive quantitative microPET imaging. This will allow us to better assess improvements in activities of our prostate specific promoter in the context of our gutless adenovector system.

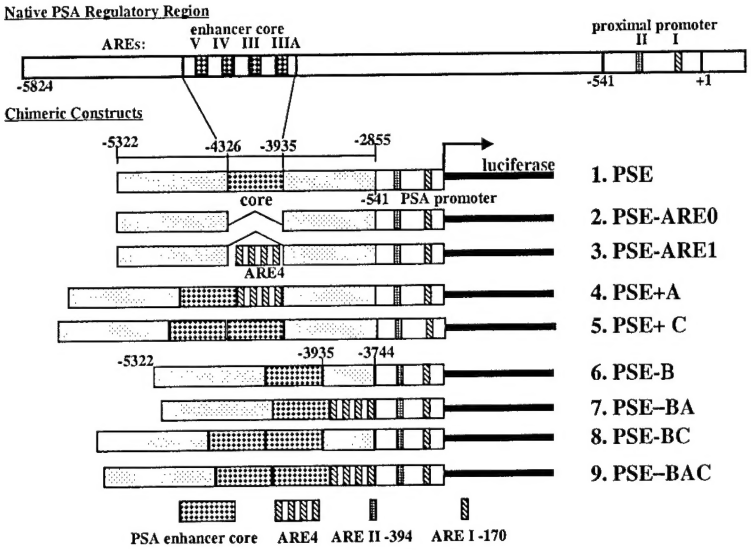

Figure 2. Chimeric PSA enhancer constructs.

Task 1.1. *Evaluation of chimeric PSA promoters.*

To overcome the low expression and tissue specificity of PSA promoter, we systematically manipulated native PSA promoter/enhancer sequences (Figure 2). The best promoters resulted from the manipulation of the proximal 600 bp promoter, the enhancer core region at - 4.2 kb, and high affinity androgen responsive elements (AREs) which are known PSA regulatory sequences. The removal of intervening sequences (- 3744 to - 2866) to position the enhancer elements closer to the proximal promoter significantly improved transcriptional activity and tissue specificity. Our best promoter, PSE-BC-luciferase construct, increased activity 19-fold when compared by transfections in androgen-dependent LNCaP cells, in the presence of 1 nM of synthetic androgen R1881 (Figure 3). Most active chimeric promoters, including PSE-BC, retained tissue specific activity when tested in the presence of 1nM of R1881 (Figure 4). The same set of constructs showed no activity in MCF-7 cells (breast cancer cell line) and HeLa cells (cervical cancer cell line). This tissue specificity is not just due to androgen receptors in LNCaP cells, since MCF-7 cells also contain low levels of androgen receptor. While optimizing our gutless adenovector, we demonstrated that the improved activity and tissue specificity of PSE-BC promoter can be retained in a first generation adenovector and is therefore feasible for further *in vivo* gene transfer studies in prostate cancer xenograft models (Figure 5).

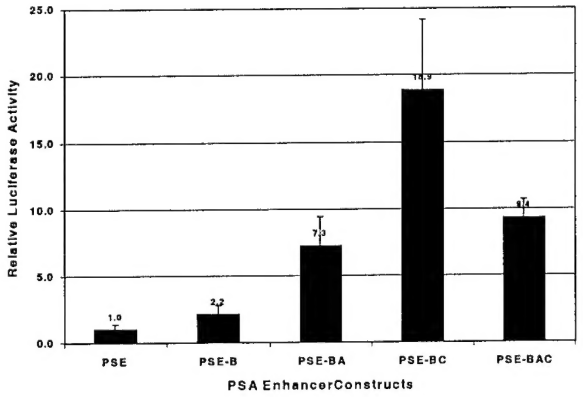

Figure 3. Activities of chimeric PSA enhancer constructs in LNCaP cells.

Task 2. *Evaluation of anti-tumor effects of TGF-β2 antisense in vitro and its contribution to reversal of immune suppression.*

In our previous progress report, we described the efficacy of our TGF-β2 phosphorothiate oligonucleotide antisense in inhibiting TGF-β2 expression in prostate cancer cell lines. Although the exact mechanism of action of TGF-β in tumor cells is still under investigation, our purpose in down-

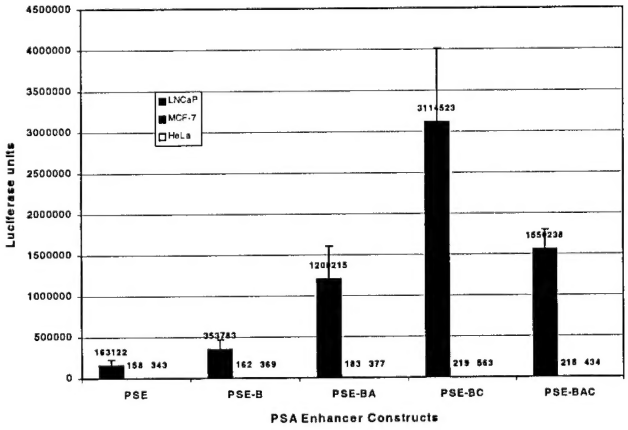

Figure 4. Comparison of prostate specificity of chimeric constructs in LNCaP and other non-prostate cells.

regulating this highly expressed growth factor in prostate tumors is to eliminate this immune suppressive and cancer promoting factor to prevent its interference in our immune-based gene therapy. In summary, we were able to achieve 63-87% inhibition of TGF-β2 production in the prostate cancer cell lines tested. The effects of TGF-β2 antisense in prostate tumor cells were evaluated *in vitro*. We observed TGF-β2 antisense-mediated growth inhibition on two prostate tumor cell lines tested (e.g., 23% growth inhibition in CL1 cells after 48 h of antisense treatment). Our *in vitro* studies also indicate that the down-regulation of TGF-β2 in prostate tumor cells may regulate the expression of TGF-β receptors. Our preliminary *in vitro* study to demonstrate the immune suppressive effects of TGF-β2 secreted by prostate cancer cells on PBL, showed that supernatants of CL1 or PC3 cells transfected with TGF-β2 antisense were able to induce 140-180% higher PBL proliferation than controls. Once validated *in vivo* in an appropriate prostate tumor model, our TGF-β2 antisense may be an important adjuvant treatment to reverse cancer immune suppression and contribute to the development of a more effective immunotherapy for prostate cancer.

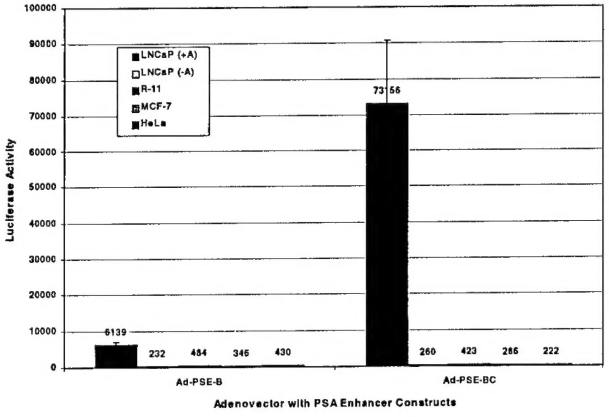

Figure 5. Use of adenovector to evaluate new PSE promoter construct.

Task 3. *Evaluation of anti-tumor effects of Apo2L/TRAIL in vitro.*

We investigated the potential use of TNF-α as an anti-cancer agent capable of inducing apoptotic tumor cell death. TNF-α is one of the most potent anti-tumor cytokine involved in direct cytotoxicity of tumor cells and immune modulation activity. However, the use of TNF-α has been limited by its systemic toxicity at therapeutic doses (18). To minimize potential toxicity and increase safety of our gene therapy, we decided to evaluate the efficacy of Apo2L/TRAIL, a TNF family member with more selective cytotoxicity against tumor cells (19,20). Our preliminary results show enhanced and tumor-selective Apo2L/TRAIL-mediated cytotoxicity using much lower concentrations than TNF-α (up to100-fold). Our results confirmed that the prostate cancer cell lines CL1, PC3, DU-145, and LNCaP are resistant to Apo2L/TRAIL at concentration of 100 ng/ml, but the killing activity could be reversed and synergized by sensitization of tumor cells using sub-clinical concentrations of actinomycin D. *In vitro* studies show that pre-treatment with 100 ng/ml actinomycin D for 24 h followed by 100 ng/ml Apo2L/TRAIL for 24 h induce a synergistic apoptotic effect in androgen-independent prostate cancer cell lines CL1, DU-145, PC3 and androgen-dependent LNCaP cells up to 89 % (Figure 6). The apoptotic effect of the combination treatment was significantly higher than corresponding mono-therapies. The potential use of Apo2L/TRAIL gene therapy is being evaluated for *in vivo* studies. We are generating gutless adenovector to deliver Apo2L/TRAIL driven by CMV promoter and by improved PSA promoter/enhancer (Figure 7). The efficacy and specificity of Apo2L/TRAIL gene expression driven by prostate specific promoter delivered by gutless adenovector will be evaluated in prostate and non-prostate tumor cell lines *in vitro* and in prostate cancer xenograft models *in vivo*.

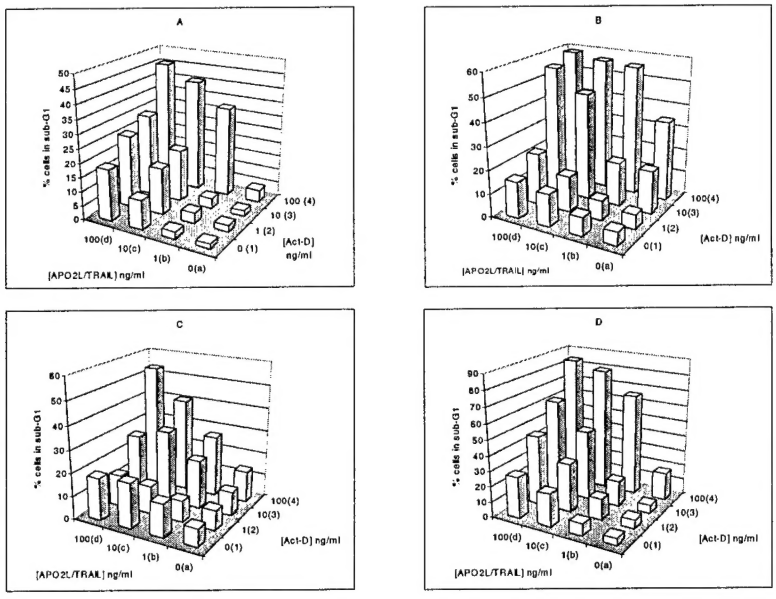

Figure 6. Apoptosis analysis in (A) CL1, (B) LNCaP, (C) DU-145, and (D) PC3

Key Research Accomplishments:

- Optimization of gutless adenovirus constructs for stabilization of the viral genome structure and improved packaging efficiency.
- Generation of chimeric PSA promoter/enhancer with improved transcriptional activity and tissue specificity.
- Development of a TGF-β2 phosphorothioate oligonucleotide antisense that effectively inhibit production of TGF-β2 in prostate cancer cell lines tested.
- Use of Apo2L/TRAIL, a TNF-α related cytokine, achieved selective cytotoxicity in our highly aggressive androgen independent CL1 prostate cancer cell line.

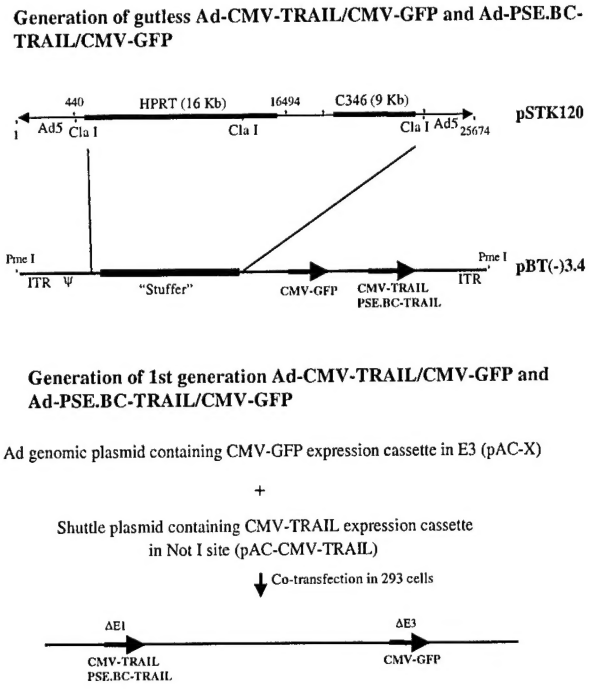

Figure 7. Scheme of first generation and "gutless" Ad containing CMV-TRAIL/CMV-GFP and PSE.BC-TRAIL/CMV-GFP.

Reportable outcomes:

1. Manuscript: "Tumor Necrosis factor-related apoptosis inducing ligand (TRAIL) for treatment of prostate cancer: first results and review of the literature" Ophoven et al. Prostate Cancer & Prostate Dis. 2: 227-33, 1999.
2. Manuscript: "Androgen deprivation induces selective outgrowth of aggressive hormone-refractory prostate cancer clones expressing distinct cellular and molecular properties not present in parental androgen-dependent cancer cells" Tso et al. Cancer J. Sci. Am. 6: 220-33, 2000.
3. Manuscript: "CL1-GFP: an androgen independent metastatic tumor model for prostate cancer" Patel et al. J. Urol. 164: 1420-5, 2000.
4. Manuscript: "Chimeric PSA enhancers exhibit augmented activity in prostate cancer gene therapy vectors" Wu et al. Submitted to Human Gene Ther.
5. Manuscript: "Actinomycin D and gemcitabine synergistically sensitize androgen-independent prostate cancer cells to Apo2L/TRAIL-mediated apoptosis" Zisman et al. in preparation
6. Manuscript: "Effects of TGF-β2 antisense in prostate cancer cells" Paik et al. in preparation.
7. Patent application on CL1 and CL2 cell lines, and CL1-GFP tumor model.
8. Patent application in progress on chimeric PSA promoter/enhancer construct.

Conclusions:

The development of a safe and efficient gene delivery vector is essential for effective prostate cancer gene therapy. We are in the process of generating gutless adenovector to deliver cytotoxic genes under control of improved prostate-specific promoter developed in our laboratory. The optimization of our gutless adenovector DNA backbone involved the replacement of 20 kb human Ori fragment by a 25 kb human HPRT plus C346 cosmid sequences (from pSTK120) for stabilization of the viral genome structure and improved packaging efficiency. We expect to improve *in vivo* transgene expression using our gutless adenovector system and to decrease toxicity using our prostate specific promoter, thus limiting the effects of cytotoxic genes only to prostate tumor cells. Our *in vitro* studies show the feasibility of combining targeted cytotoxicity with adjuvant treatment to block over expression of immune suppressive factors. We made significant progress in developing a TGF-β2 phosphorothioate oligonucleotide antisense that effectively blocks the production of TGF-β2 in prostate cancer cell lines tested. We also have demonstrated superior cytotoxic effect of Apo2L/TRAIL to target tumor cells *in vitro*. Our on going *in vivo* studies will further evaluate and validate the efficacy and safety of our combined immune-based approach to target and eradicate prostate cancer cells.

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Chimeric PSA Enhancers Exhibit Augmented Activity in Prostate Cancer Gene Therapy Vectors

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Summary
The native PSA enhancer and promoter confer prostate-specific expression when inserted into adenovirus vectors capable of efficient *in vivo* gene delivery, although the transcriptional activity is low. By exploiting the fundamental properties of the natural PSA control regions we have improved the activity and specificity of the prostate specific PSA enhancer for gene therapy applications. Previous studies had established that Androgen Receptor (AR) molecules bind cooperatively to AREs in the PSA enhancer core (-4326 to –3935) and act synergistically with AR bound to the proximal promoter to regulate transcriptional output. To exploit the synergistic nature of AR action we generated chimeric enhancer constructs by 1) insertion of 4 tandem copies of the proximal AREI element, 2) duplication of enhancer core, or 3) removal of intervening sequences (–3744 to -2855) between the enhancer and promoter. By comparing to baseline construct, PSE, containing the PSA enhancer (-5322 to –2855) fused to the proximal promoter (-541 to +12), the 3 most efficacious chimeric constructs, PSE-BA (insertion or ARE4), PSE-BC (duplication of core) and PSE-BAC (insertion of core and ARE4), are 7.3-, 18.9-, and 9.4-fold higher respectively. These chimeric PSA enhancer constructs are highly androgen inducible and retain a high degree of tissue discriminatory capability. Initial biochemical studies reveal that the augmented activity of the chimeric constructs in vivo correlates with their ability to recruit AR and critical co-activators in vitro. The enhanced activity, inducibility and specificity of the chimeric constructs are retained in an adenoviral vector.
Keywords: PSA promoter and enhancer, androgen responsive element, adenoviral vector, tissue specificity.

Introduction

We have implemented a study to improve the design of therapeutic vectors for prostate cancer, a disease affecting over 300,000 men and causing 40,000 deaths per year.[1] Gene therapy protocols have been hampered by low expression and a lack of specificity, resulting in concerns of efficacy and safety. One approach for circumventing these problems in prostate cancer gene therapy is to develop a prostate-specific vector with greatly enhanced transcriptional activity, which retains a high degree of tissue specificity. The concept is that if the vector is able to exclusively restrict therapeutic gene(s) expression to the prostate, then the vector, when inadvertently delivered to non-targeted organs, will remain transcriptionally silent. This targeted approach should reduce potential side effects of cytotoxic gene therapy.

Normal, hyperplastic and malignant prostate epithelia specifically express the kallikrein protease Prostate Specific Antigen (PSA).[2] Serum levels of PSA are used clinically as a marker for diagnosis and management of prostate cancer.[3,4] The PSA gene regulatory regions are prime candidates to direct prostate-specific expression.[5-7] The PSA regulatory region (figure 1A) includes a proximal promoter (600 base-pairs (bp) upstream of the transcription initiation site) contains a TATA box and two functionally important AREs (AREs, binding sites for the transcription factor AR [8-12]). This promoter alone exhibits tissue specific expression in vitro in cell based assays, although it is insufficient to direct prostate specific expression in vivo.[13,14] It has been established that a 6 kilo-bases (kb) region upstream of the gene contains all of the genetic information to direct prostate-specific expression in transgenic mouse experiments.[13,15]

Three important successive studies narrowed down smaller upstream enhancer regions within the 6 kb that conferred prostate specific PSA gene expression.[5-7] An 1.6-kb enhancer (-5322 to –3738) containing putative recognition sites for AR, AP-1, and c-fos[5] and a smaller 822-bp enhancer fragment[7] (-4757 to –3935) were able to achieve maximal activity comparable to the entire 6 kb PSA regulatory region. The importance of this region was highlighted by the identification of an androgen-inducible DNase hypersensitive site (DHS II) centered at –4.2kb, mapped in chromatin from LNCaP prostate cancer cells.[6] Functional analyses of the DHS II region revealed a minimal 440-bp core enhancer (-4324 to –3884), which provided strong enhancer activity and contained a high affinity ARE (AREIII[6]). Subsequent studies demonstrated that the core enhancer contained at least four non-consensus AREs with variable binding affinity for AR,[16] all of which contribute to the synergistic activation of PSA gene expression. [16]

Studies utilizing combinations of native PSA regulatory regions to drive therapeutic genes[17, 18, 19] in cell culture studies have demonstrated approximately a 10 fold prostate tissue discriminatory activity and androgen inducibility. The expected therapeutic response of tumor cell ablation was observed in PSA expressing prostate cancer cells. Validating these prostate-specific therapeutic responses in animal models and patients will require a vector capable of efficient in vivo gene transfer to deliver the PSA promoter driven therapeutic transgenes. Recombinant adenovirus vectors (Ads) have the capacity to deliver genes in vivo and intra-prostatically,[20] and they have been utilized in several on-going human prostate cancer clinical trials.[21,22] Two Ads with either the entire 5.8-kb (-5825 to +12) PSA regulatory region or 1.6-kb (-5322 to –3738) enhancer linked to the proximal PSA promoter (-541 to +12), were able to direct either prostate-specific expression of cytotoxic HSV TK[19] or viral E1A[22] to induce lytic viral

replication. The efficacy, tissue specificity and safety of these therapeutic approaches were not fully evaluated in vivo. Our studies illustrated that the native PSA enhancer and promoter (–5322 to -2855 and –541 to +11 of PSA gene) inserted into Ad could direct tissue specific and 10-fold androgen-inducible expression in LNCaP cells, but its transcriptional activity is lower than the constitutive CMV promoter by 50-fold or more (data not shown).

A basic understanding of transcription regulatory mechanisms is necessary to devise logical approaches to improve the activity and specificity of the PSA enhancer and promoter. Although the precise mechanism of PSA enhancer function is unclear, recent results clearly demonstrated that the compact and functionally important enhancer core (-4366 to –3874) contains a cluster of four non-consensus AREs[16] of two distinct classes (I and II[23]). The class I and II sites each have distinctive nucleotide variations within the ARE that confer differential binding patterns and functions to AR.[23] Although the isolated class I sites have higher AR binding affinity than class II sites, binding to class II site facilitates adjacent class I site function resulting in cooperative AR binding to the tandem AREs.[23] This cooperative binding contributes to AR-mediated synergistic transcriptional activation.[16, 23] These studies lend support to the current idea that an enhanceosome composed of multiple activators engage in specific, cooperative and often combinatorial interactions, which lead to the assembly of a stable nucleoprotein structures that govern the specificity of transcription. Thus, it is plausible that AR anchors such a structure onto the PSA enhancer.[24-27]

In an attempt to harness the synergy and cooperative nature of AR bound to the transcriptional enhancer element, we undertook a systematic combinatorial manipulation of native and artificial enhancer elements. In one approach, we fused an artificial regulatory element (ARE4, figure 1B) comprising 4 tandem copies of a high affinity class I ARE (derived from 15 bp AREI site of PSA promoter, [10]) to the native PSA enhancer and promoter construct (PSE). In a second strategy we duplicated the core enhancer. These 2 approaches in conjunction with the removal of intervening sequences (–3744 to -2855) to position the enhancer elements closer to the proximal promoter have resulted in greatly augmented transcriptional activity and tissue specificity. The several improved chimeric constructs mediate significantly higher (>10 fold) activity versus the PSE. More importantly, the augmented activity of our PSA enhancer construct is validated in a therapeutically relevant Ad.

Results

Concept of the Chimeric Enhancer

The goal of our study was to create prostate specific gene therapy constructs with high activity and exquisite tissue specificity. The PSA enhancer contains a natural androgen inducible sub region centered 4.2-kb upstream from the transcription startsite (figure 1B). We reasoned that by combining this region with an artificial enhancer, or by duplicating the region, we could create a chimera with increased activity while retaining prostate specificity. To create the artificial enhancer, we multimerized the ARE I site found in the proximal PSA promoter region (figure 1A). We hypothesize that this ARE4 should function synergistically with the enhancer core when transposed directly adjacent to it.

The parental construct, PSE, consisted of the 2.4 kb enhancer fragment (-5322 to -2855) and the proximal promoter region from –541 to +12, upstream of a luciferase gene. PSE

was chosen to serve as the baseline construct because the 2.4 kb enhancer fragment generates the maximal transcriptional and androgen responsive activity, comparable to the entire 6-kb regulatory region of the PSA gene.[6] The androgen inducible core region bearing four natural AREs is defined by two flanking restriction sites, BstEII and NcoI (-4326 to –3935). We constructed the following reporter gene variants: one lacking the core enhancer (PSA-ARE0); one containing ARE4 in place of the core (PSE-ARE1); one in which ARE4 was inserted immediately downstream of the enhancer core at –3935 (PSE+A); and finally, a construct bearing a duplicated core region, where an additional copy of the core was placed upstream at –4326 (PSE+C). To further augment enhancer activity we postulated that removing the sequence between the enhancer and promoter would increase activity further. This set of constructs is termed the PSE-B series. Figure 1B is a schematic representation of all the constructs tested.

Activities of chimeric constructs

The constructs were tested initially in cell culture transfections by analyzing three criteria: transcriptional activity, androgen inducibility and cell specificity. Relative activities of constructs were compared by transfections in LNCaP cells (an AR+, PSA+ prostate cancer cell line) in the presence of 1nM synthetic androgen R1881. PSE exhibited low to modest absolute activity in LNCaP cells, approximately 1 to 2 % that of CMV (data not shown). Figure 2A displays the activities of all constructs relative to PSE (= 1). The activity was enhancer dependent because removal of the core region abolished transcription to 10% of PSE. Addition of ARE4 in place of the core did not restore activity. However, the positioning of ARE4 adjacent to the core (PSE+A) led to a substantive 3.3-fold increase in activity over the baseline PSE. Duplication of the core in PSE+C also enhanced activity by 1.9 fold. In summary the data bore out the premise, that ARE4 in combination with the core would synergize with the natural region to raise PSA enhancer activity.

Figure 2B shows that removing the intervening DNA augments the activity of all the constructs from the parental to the chimeric constructs. Removal of sequences –3744 to –2875 from PSE raised the activity by 2.2 fold (PSE-B). Further combinatorial modifications drastically enhanced activity 7.3, 18.7 and 9.4-fold higher than PSE in PSE-BA, PSE-BC and PSE-BAC, respectively. Recapitulating the stimulatory effects by removal of intervening sequences, PSE-BA and PSE-BC achieved an additional 2.2- and 9.8-fold augmentation over their extended counterparts PSE+A and PSE+C.

Androgen Inducibility and Tissue Specificity in Chimeric Constructs

The chimeras exhibited greatly increased androgen inducibility. Figure 3A presents the androgen induction of all the constructs, calculated by comparing the luciferase activity of LNCaP cells transfected in the presence of 1nM R1881 over those transfected in the absence of androgen, i.e., cells maintained in charcoal-stripped serum containing media. The higher activities of the chimeric constructs are illustrated by enhanced androgen responsiveness over the parental PSE. This point is best illustrated by PSE-BAC, PSE-BC and PSE-BA, the three most active constructs. They exhibited the highest androgen inducibility from 96- to 201-fold. Although the absolute basal activities of all constructs were similar in any one experiment, variations were observed in different experiments and may have been due to difficulties in depleting all androgen from the

media (data not shown). The variation of basal activity also affects the fold inducibility index since it is calculated by the ratio of androgen-induced over basal activity. Figure 3B shows the results from a separate transfection experiment, where to validate that PSE-BAC retained normal responsiveness it was compared with the parental PSE for androgen inducibility. As the androgen concentration in the culture was increased the activation profile of the chimeric and parental constructs paralleled each other. The activities of the constructs were negligible at 0.1 nM, gradually increased from 0.1 to 1 nM, and peaked at 10 nM. PSE-BAC induced 232 fold at 1 nM R1881, compared with 16.7 fold for the parental, as shown on the vertical axis of the graph. These results argue that the augmented transcriptional activity of the chimeras is indeed due to increased androgen responsiveness as predicted by our original hypothesis.

All of the chimeras retain cell-type selectivity in culture. Our most active constructs were compared via side by side transfections into either MCF-7 cells, a breast cancer line, HeLa cells, a cervical carcinoma line, and LNCaP cells, all in the presence of 1nM R1881. All constructs exhibited negligible activity in the presence of 1nM R1881 in MCF-7 and HeLa cells (figure 4A). However, under the same androgen-containing growth conditions in LNCaP cells, the parental and chimeric constructs elicited 3 to 4 order of magnitude higher activities, respectively. For example, the activity in LNCaP over MCF-7 and HeLa for PSE is 1032- and 476-fold higher, and for PSE-BC the activity is 14221- and 5532-fold higher respectively. This tissue specific activity is not due solely to the presence of AR in LNCaP since MCF-7 cells contain low levels of AR[9,28] but fail to activate the reporters.

To investigate the contribution of AR to the tissue specificity of PSA enhancer constructs, we utilized a HeLa cell line engineered to stably express epitope-tagged AR (fAR-HeLa[16]) at a two fold higher level than LNCaP. Constructs transfected into fAR-HeLa cells were androgen responsive (figure 4B) but to a much lower extent than in the prostate cancer cell line LNCaP (figure 3A). The observed induction of 3.2- to 12.1-fold in fAR-HeLa is at least 10 times lower than the activity observed in LNCaP cells, with PSE-BA exhibiting the highest androgen inducibility. These results suggest that fAR-HeLa cells are lacking prostate specific factors that amplify the androgen responsiveness in LNCaP. Alternatively, Hela cells may process transcriptional suppressors not present in LNCaP.

Recruitment of AR and Co-activators Correlates with Enhanced Activity

The augmented activity of the constructs in vivo correlates with their ability to recruit AR and critical co-activators in vitro (figure 5). An immobilized template approach was applied to study recruitment of AR and co-factors in vitro in LNCaP extracts. The relevant regulatory regions were biotinylated and immobilized in equal amounts on streptavidin magnetic beads. The immobilized templates were then incubated in LNCaP nuclear extracts. Unbound protein was removed by washing, and the bound proteins were analyzed by immunoblotting with antibodies against AR and SRC-1. Little or no AR was detected bound to PSE-ARE0 in any of several repetitions of this experiment. The immunoblots show, however, that PSE and PSEARE1 recruit low but similar levels of AR, as expected as they contain similar numbers of AREs. Remarkably PSE+A, and PSE+C recruit substantial amounts of AR again as expected. The blot shows

that PSE+C can recruit the AR co-activator SRC-1 although longer exposures showed that PSE+A recruits about half as much.

The Chimeric Enhancers Retain Activity in the Context of Adenoviral vectors

When selected constructs were inserted into Ads they retained regulated transcriptional activity and cell type selectivity. We chose to compare the activity of our most efficacious construct PSE-BC to that of PSE-B because a previously described prostate-specific Ad, currently being evaluated in a clinical study,[22] comprises almost the precise enhancer and promoter sequences as PSE-B. The recombinant Ads were generated by in vivo homologous recombination.[29] A schematic representation of the Ad was shown in figure 6A. Prostate and non-prostate cells were equivalently infected with an MOI 5 (5 infectious units/cell) of AdPSE-Bluc or AdPSE-BCluc. The similarity in infection efficiency was evident by similar levels of intracellular viral DNA measured by Southern blotting (figure 6B). In the presence of androgen in LNCaP cells, AdPSE-BCluc activity, as reflected by luciferase units, was 11.9-fold higher than AdPSE-Bluc (figure 6C). The androgen inducibilities of AdPSE-Bluc and AdPSE-BCluc were 27- and 281-fold respectively. Both constructs retained strict tissue specific activity as expression in R-11 (a renal carcinoma line),[30] MCF-7 and HeLa cells were negligible despite our observation that these 3 lines are all readily infected by Ad (data not shown). These results correspond well with the augmented activity, androgen responsiveness and tissue-discriminatory capability observed in transfection analyses. This work should pave the way for the development of prostate-specific gene therapy vectors with improved activity and safety parameters for clinical applications.

Discussion

Specific transcriptional targeting strategies are beneficial in gene therapy applications because restricting the expression of toxic therapeutic genes to malignant prostate cells provides additional safety measures over constitutive expression strategies. However, the transcriptional output from the native PSA enhancer and promoter, and from most highly regulated tissue specific promoters, is much lower than from strong constitutive viral promoters such as CMV. Our goal was to manipulate promoter and enhancer activity to achieve both high and specific gene expression to achieve safe and effective gene therapy in vivo.

Over the past decade well-defined mechanisms of gene activation have emerged from studies in the transcription field. Cooperative binding of multiple sequence-specific transcription factors to the proximal promoter and distal enhancer of a gene, and subsequent recruitment of co-activator proteins, leads to the assembly of a large general transcription initiation complex over the start site of a gene.[25,31] Enhancer mediated transcription activation is likely achieved by further cooperative interactions between the enhancer- and the promoter-complex, via looping out the intervening DNA. The strong cooperative regulation of initiation complex assembly is believed to be responsible for tissue-specific gene expression.[32] The precise mechanism of how the PSA regulatory elements orchestrate an accurate and prostate-specific transcription program is incompletely understood, but one essential component of enhancer activation function is mediated through cooperative AR-AR interactions.[16,23] Studies have also pointed to the

involvement of both prostate-specific and ubiquitous transcription factors in cell specific regulation. [5,6,33]

Guided by established principles of transcription regulation, we manipulated the known PSA regulatory components, namely the proximal 600-bp promoter, the enhancer core centered at –4.2kb, and high affinity AREs, to achieve augmented prostate specific expression. The three strategies we utilized, 1) insertion of ARE4, 2) duplication of the enhancer core element, and 3) removal of intervening sequences between the enhancer and promoter, all increased transcription activity and androgen inducibility. The manipulations resulted in 3 highly active constructs, PSE-BA, PSE-BC and PSE-BAC, exhibiting 7.3-18.9-fold higher activity, and 96- to 201-fold higher androgen inducibility, relative to our baseline PSE construct. Moreover, the constructs retained a high degree of tissue discriminatory capability such that their expression is much higher in LNCaP androgen responsive prostate cells than non-prostate, AR expressing cells.

Juxtaposing the synthetic ARE4 immediately 3' to the enhancer core in our chimeric constructs (PSE+A, PSE-BA and PSE-BAC) achieved the desired effect of enhanced activity over the baseline PSE construct. The ARE4 synergizes with the enhancer core element to activate transcription. The increase was synergistic because the core or ARE4 on its own elicited only modest or no activity respectively (i.e. activity of PSE to PSE-ARE0 is 10 and PSE-ARE1 to PSE-ARE0 is 1, see figure 2C). However, the combination of core and ARE4 (PSE+A to PSE-ARE0) resulted in 33 fold enhancement which is greater than an additive effect. One step in the mechanism of activation involves increased binding of AR to the chimeric enhancer element (see figure 5), which in turn likely induces interactions of a co-activator, such as SRC-1.

Although the PSE-BAC construct contains the ARE4 element in addition to a duplicated enhancer core, its activity is slightly lower than PSE-BC. It is unclear whether the deletion of an additional 190-bp (-3935 to –3744) in PSE-BC, (i.e., the sequence replaced by ARE4 in PSE-BAC see methods section for details), contributes to the lower than expected level of activity. A binding site for a novel prostate specific Ets-family transcription factor, PDEF, is located at –3848 in the midst of the deleted sequences.[33] Although transient expression of a PDEF augmented PSA promoter activity in prostate cells and non-AR expressing cells, [33] the precise contribution of PDEF to PSA promoter function requires further clarification.

The PSA enhancer core region (-4326 to –3955) mediates transcriptional activation in a prostate-specific manner, working in concert with the proximal promoter. This point is illustrated by results in figures 2D and 4A, where an additional copy of the enhancer core greatly increased transcriptional output in androgen responsive prostate cells but exhibited no detectable effect in non-prostate cells. Although multiple AREs within the enhancer core contribute greatly to its activity, [16] there are almost certainly undefined components within this element that govern prostate-specific transcriptional activity.[5,6,33] This point is emphasized by the observation that replacing the deleted core (PSE-ARE0) with the ARE4 element (PSE-ARE1) did not rescue its activity. Therefore, we conclude that the chimeric constructs achieve their effect by combining the transcription stimulatory activity of ARE4 with the prostate specificity of the core element, a point substantiated by our various constructs.

A similar theme has emerged in two recent studies, validating the concept that multimerization of key promoter or enhancer elements improves prostate-specific

transcriptional activity. [34,35] Constructs with three copies of the androgen responsive region (ARR, -244 to-96) of the rat probasin promoter that contained multiple class I and II AREs[23] exhibited about 20-fold higher activity than when a single ARR was linked to minimal promoter reporter construct.[34] Studies presented in Latham and colleagues' paper involved constructs that multimerized the larger 1.4 kb PSA enhancer fragment (-5322 to –3869) and linked it to the 600 bp PSA proximal promoter. The most active construct (GL3 E2 PSA) exhibited an approximately 4-fold increased activity over a construct with a single enhancer fragment (GL3-E-PSA) in LNCaP cells. This approach is similar to our strategy for generating PSE-BC, except our duplicated sequences (-4326 to –3935) are more than 1kb smaller. Accurate comparison of their results to our study is difficult because the methodology and the baseline constructs are different. However, a relative comparison of activity indicates that duplication of the much smaller 390 bp enhancer core appeared to be more effective than duplicating the larger 1.4-kb fragment. As illustrated in figure 2D, PSE-BC activity is 18.9-fold higher than PSE and 8.5-fold higher than PSE-B, which contains an enhancer (-5322 to –3744) region similar to the GL3-E-PSA (-5322 to –3869) construct. Interestingly, 3 copies of the 1.4-kb enhancer resulted in diminution of activity. [35] We observed the same phenomenon as either 3 or 4 copies of the enhancer core diminished activity below PSE (data not shown). Mechanistically, it is interesting to speculate that too many binding sites for transcription factors might disperse limiting factors or co-activators to locations that reduce cooperative interactions and functionality.

Demonstrating the improved activity and specificity of our modified PSA enhancer plasmid constructs are retained in an Ad is an important step towards achieving successful prostate cancer targeted gene therapy. Since Ads are capable of efficient gene transfer in vivo, the next important step is to demonstrate the efficacy of gene transfer in relevant animal models of human prostate cancer. Due to a shortage of PSA producing, androgen responsive human prostate cancer lines or animal models, LNCaP cells have been widely utilized to evaluate expression of PSA derived expression vectors. Recently, several novel human prostate cancer xenografts have been propagated which accurately retain key clinical characteristics of human disease.[36,37] Namely, both LAPC4 and LAPC9 require androgen for growth, synthesize PSA, express non-mutated AR, progress to androgen independence upon androgen withdrawal and develop metastatic disease[36] (CL Sawyers personal communication). Our preliminary results indicated that AdPSE-BCluc mediates high expression in LAPC9 xenografts after intratumoral Ad injection (data not shown).

To implement a quantitative and non-invasive method capable of monitoring transgene expression in living animals repetitively would be a crucial step toward validating the efficacy of prostate-specific or any gene therapy strategy. A positron emission tomography (PET) based on Herpes Simplex Virus 1 Thymidine Kinase (HSV1-TK) trapping of positron-labeled substrates has been demonstrated to be a sensitive and quantitative modality to image the location and magnitude of Ad mediated HSV1-TK gene expression in living animals.[38,39] The advent of better human prostate cancer models, advanced gene expression imaging technology and improved prostate specific vectorology could lead to an era where a safe and effective prostate cancer gene therapy protocol is a reality.

Materials and methods

Cell cultures

The LNCaP, MCF-7 and R-11 cells were grown in RPMI 1640(Gibco-BRL, MD) with 10%FBS (Gemini Co., Woodlands CA) and 100 units/ml of penicillin and streptomycin. The R-11 renal carcinoma cell line was derived from a patient with kidney cancer.[30] HeLa cells and 293 cells were grown in DMEM (Gibco-BRL, MD) supplemented with 10% FBS and penicillin and streptomycin. 293 cells, grown as monolayers, were obtained from Microbix Biosystems Inc. (Toronto, Canada). 293 suspension cultures, used for large-scale propagation of Ad, were maintained at concentration of $4x10^5$ cells/ml of S-MEM (Gibco-BRL, MD) supplemented with 5% FBS and penicillin and streptomycin.

Androgen induction analyses were accomplished by replacement of FBS containing media to media containing 10% charcoal/dextran stripped FBS,[7] 1 day and 4 hrs before transfection or infection. Changing the media twice was to ensure removal of trace amount of androgen in media. Methyltrienolone, R1881, (NEN Life Science Products, Boston MA) dissolved in ethanol was added to the 10% charcoal stripped FBS containing media at specified concentration after transfection or infection.

Plasmid constructs:

The starting baseline construct pPSE were constructed in a modified pBS II SK+ (Strategene Inc.) where the Asp718 site is converted to a NotI site. By design the insertion of a NotI flanked PSA enhancer/promoter expression cassette (spanning from upstream enhancer to the end of polyA element) removes all restriction sites between Asp718 to NotI of pBS. We then inserted multiple cloning sites encoded by oligonucleotides (HindIII, EcoRV, EcoRI, Asp718, SwaI and SalI) between the PSA enhancer/promoter and SV40 polyA to facilitate insertion of different transgenes. The PSA enhancer/promoter fragment, 5' NotI to 3' HindIII derived from plasmid PSAR2.4k-PCPSA-P-Lux,[7] contains PSA regulatory region from -5322 (XbaI) to –2855 joined to proximal promoter –541 (BglII) to +12 (HindIII). The SV40 polyA, 5'HindIII to 3'NotI, fragment is derived from pACCMVpLpASR.[40] The ARE4 157 bp synthetic element was generated by first ligating and multimerization of paired oligonucleotides containing the 15 bp AREI. The paired primers and PCR were used to incorporate the designed terminal restriction sites and the precise nucleotide sequence of ARE4 was confirmed. The ARE4 sequence is flanked by two NcoI sites and also contained a BstEII and a BamHI site immediately 3' of the 5' NcoI site (see figure 1B). Insertion of this ARE4 synthetic sequence into the unique NcoI site (-3935) of pPSE resulted in two orientations of PSE+A. Both orientations of PSE+A have comparable activities (data not shown). The orientation of PSE+A where the BstEII site of ARE4 is more distal to –3935 NcoI (PSE+A/-) was retained and analyzed in all subsequent studies. The control PSE-ARE0 was generated by cutting PSE+A/- with BstEII and re-ligation which remove the enhancer core region. The other orientation of PSE+A where the BstEII site is immediately 3' to –3935 (PSE+A/+) was utilize to generate the enhancer core fragment from –4326 to –3935 flanked by two BstEII sites and pPSE-ARE1. Insertion of this BstEII enhancer core fragment (and its multimers) into the unique BstEII at –4326 of pPSE resulted in PSE+C construct (and 3 or 4 copies of core). The control construct

pPSE-ARE1 was generated by re-ligation of BstEII restricted pPSE+A/+, resulted in ARE4 replacement of the enhancer core.

The PSE-B series of plasmids were generated by cutting with BamHI and BglII, followed by re-ligation since the two enzymes have compatible ends. This manipulation of PSE, PSE+C resulted in the removal of enhancer sequence from –3743 (BamHI) to –2855, thus linking –3744 directly to –541 (BglII) of proximal promoter of PSE-B and PSE-BC. The same BamHI to BglII deletion in PSE+A resulted in extended sequence removal, -3935 to –2855 in PSE-BA, because of the presence of a BamHI site in the ARE4 sequence. PSE-BAC was generated by insertion of the BstEII flanked enhancer core fragment into PSE-BA. The HindIII to EcoRI luciferase gene derived from pSP-luc+ (Promega Co.) was inserted into all the PSA enhancer/promoter constructs for activity assays.

Transfections and DNA analysis:

$5x10^4$ HeLa, MCF-7 or R11 cells or 1 $x10^5$ LNCaP cells were plated into 24-well plates 48 hours prior to transfection. The activity of the different constructs, each bearing a downstream luciferase reporter gene, was assayed by transfection using the lipid reagent Lipofectamine Plus (GibcoBRL Co.) or Tfx 50 (Promega) into cells. Assays in quadruplicate were performed according to manufacturer's guideline and the two reagents gave comparable results. 1 day after transfection, the cells were harvested and lyzed in 100ul of 1x passive lysis buffer according to manufacturer's instruction (Promega Co., Chicago IL). 10ul of cell lysate from each transfection was measured by luminometry (luminometer: Monolight 2010, Analytical Luminescence Laboratory Co.). Relative luciferase activity was calculated in reference to PSE which was assigned a value of 1 (100,000 to 200,000 luciferase activity in presence of 0.1 nM R1881). We chose this designation so that the augmented activity of the chimeric constructs could be directly compared with PSE.

A modified Hirt DNA isolation procedure[41,42] was used to obtain low molecular weight DNA from cells 24 hours after adenovector infection. An aliquot of each infected cell culture was also analyzed for luciferase expression. Hirt DNA from 10^6 LNCaP cells infected at MOI 5 (virus : cell ratio = 5) was analyzed by Southern Blot using nonradioactive digoxigenin labeled luciferase DNA probe (Roche Co.). Transferred DNA on nylon membrane (Hybond, Amersham Co.) was detected by colorimetric conversion of NBT and BCIP. All procedures were performed according to manufacturer's protocols.

Recombinant adenoviruses:

To generate recombinant adenovector, the NotI luciferase expression cassettes from PSE-Bluc and PSE-BCluc were cloned into the pACCMVpLpASR[40] replacing the NotI CMV expression cassette, resulting the pAC-PSE-Bluc and pAC-PSE-BCluc respectively. The pACCMVpLpASR[40] contains Ad5 DNA from nt 1 to 454, NotI CMV expression cassette and Ad5 DNA nt 3334 to 6231. Co-transfection by $CaPO_4$ method[29] of either pAC-PSE-Bluc or pAC-PSE-BCluc with pJM17[43] and subsequent in vivo homologous recombination between the two transfected plasmids in 293 monolayer cells resulted in the emergence of respective recombinant adenovector, Ad-PSE-Bluc and Ad-PSE-BCluc. Single isolated viral plaque was expanded and screened by restriction digestion analysis of viral DNA prepared by Hirt isolation from infected cells. Confirmed viral clones were plaque-purified three successive rounds. Viral stocks were prepared by large-scale

infection of 293 suspension cultures. Virus was purified by $CsCl_2$ step gradient ultra-centrifugation, dialzyed to remove $CsCl_2$, and resuspended in 10mM Tris (pH 7.5), 10% glycerol and 10% FBS and stored at -80^{0}C. Viral stock titers were determined by plaque formation on 293 monolayer cells. [29]

Immobilized Template Pulldown Assay:

The basic concept of this assay is to compare the binding of relevant transcription factor or co-activator from LNCaP nuclear extracts to each immobilized enhancer template construct. LNCaP nuclear extracts were prepared according to Huang et al.[16] Biotinylated DNA containing the key enhancer core region from each of the specified construct was generated by PCR using a pair of primers corresponding to –4383 XXCAGACAGCATGAGGTTCATGTTCACA –4358 (5' primer, X=biotin) and –3894 GGGTGGAAGGCTCTGGCTGAACAGCGT -3920 (3'primer). Strepavidin solid support beads (30µl from Dynal Co.) were washed twice in buffer containing 10mM Tris-HCl, 1mM EDTA and 2M NaCl. Each biotinylated template DNAs were allowed to bind to the 30µl of washed strepavidin beads for 60 minutes at room temperature. The beads were washed in buffer and resuspended in 0.1M Buffer D.[44] The precise template concentration bound to beads was quantified by digestion of 5µl of beads with *HindIII*, the beads were run on a 1.5% agarose gel and the concentration estimated by comparison with standards. LNCaP nuclear extracts (NE) were pre-incubated with pGEM3 (Promega Co.), at a ratio 50µl NE to 100 ng pGEM3 for each reaction, by rotating10mins at 4^{0}C to eliminate non-specific binding. This NE was desalted and pre-cleared by passing through Micro bio spin 6 columns (Bio-Rad Co.) which had been equilibrated with 0.1M Buffer D. 100 fmol of each immobilized template was incubated with 50ul of pre-cleared and desalted NE by incubating 45mins at 4^{0}C. After binding the beads were washed twice with 200 ul of buffer containing 125 ul buffer D, [44] 15 ul 0.1 M $MgCl_2$, 10 µl 1% NP-40, 2µl of 50µg/ml BSA and 48 µl ddH_2O. Proteins bound to the solid templates were eluted directly by boiling in 2X loading dye and resolved on standard Western blot analysis. The anti-AR (Santa Cruz Co.) and anti-SRC1 (Santa Cruz Co.) antibodies were used to detect the respective protein.

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Figure Legends

Figure 1. Synthetic ARE4 element and chimeric PSA enhancer constructs. A. The sequence of ARE4 synthetic enhancer element. The 157 bases include 4 copies of ARE elements (underlined and bold) in reverse (r) or forward (f) orientation. Restriction sites at both terminii are indicated by bold and italic sequences. **B.** The native PSA enhancer and promoter region including the identified androgen response elements (ARE, binding sites for the transcription factor AR). The AREs in the enhancer core region[5-7, 16,23] and proximal promoter[5, 8-11] were defined to be functionally important. Chimeric enhancer constructs are schematically represented. PSE, the starting construct,[7] bears the 2.4-kb enhancer fragment and 541-bp promoter. Core indicates the region between the BstEII and NcoI sites and it contains at least 4 key ARE elements.[16, 23] The constructs testing the first 2 strategies are the PSE plus ARE4 (PSE+A) and PSE plus an additional copy of core (PSE+C). Controls for the effects are PSE alone, PSE lacking the core (PSEARE0) and PSE containing ARE4 in place of the core (PSEARE1). A second set of constructs, termed the PSE-B series, tests whether placing the enhancer closer to the promoter up-regulates activity. These constructs remove sequences between either -3744 to –2855 (PSE-B and PSE-BC) or –3935 to –2855 (PSE-BA and PSE-BAC).

Figure 2. Activities of chimeric PSA enhancer constructs in LNCaP cells. A. Activity of chimeric constructs. The activity of the different constructs, each bearing a downstream luciferase reporter gene, was assayed by transfection using the lipid reagent Lipofectamine Plus (Gibco) or Tfx-50 (Promega) into LNCaP cells in the presence of 1nM R1881 to activate enhancer activity. The vertical axis is relative luciferase activity where the activity of PSE was assigned a value of 1. Assays were performed in quadruplicate and the standard deviation is indicated with error bars. **B.** Activity of PSE-B series. This set combines chimeric constructs with removal of sequences between enhancer and promoter. Conditions of transfection are the same as in A.

Figure 3. Androgen Inducibility. A. The androgen inducibility of the chimeric constructs is greater than that of PSE. LNCaP cells, aliquoted into 24-well plates, were changed to androgen depleted media (RPMI + 10% charcoal stripped FBS) 24 hr. prior to transfection. After transfections, wells were replaced with the same androgen depleted media or with 1 nM R1881 added. Fold of androgen induction is calculated by the ratio of luciferase activity in androgen-containing over androgen-depleted media for each construct. **B.** PSE and PSEBAC were transfected into LNCaP cells and the indicated concentrations of R1881 were added. The vertical axis represents fold induction versus PSE measured in the absence of R1881.

Figure 4. Comparison of prostate specificity of chimeric constructs. A. Comparing parallel transfections into LNCaP and other non-prostate cells; MCF-7, breast cancer line; HeLa, cervical carcinoma line. The experiment was performed in the presence of media containing 1 nM R1881. The vertical axis represents absolute luciferase units as indicated by the numbers above the bars. The bars are in sets of three, represented from left to right by LNCaP, MCF-7 and HeLa, respectively. The low absolute activities in MCF-7 and HeLa versus LNCaP demonstrate that the enhanced activity is cell type specific in transfection assays. **B.** Activity of chimeric constructs in non-prostate fAR-HeLa cell with engineered exogenous AR expression. Conditions of experiments are as indicated in

figure 3A except fAR-HeLa cells are maintained in DMEM media +10% charcoal-stripped FBS with or without added 1nM R1881.

Figure 5. Immobilized template pulldown assays on DNA from enhancer core region of specified constructs. Each template was generated by PCR with the same pair of 5' primer (biotinylated) and 3' primer. The templates were then immobilized on strepavidin magnetic beads. 100 fmoles of each immobilized template was incubated with LNCaP nuclear extract that was pre-cleared for non-specific binding. Proteins bounded to each template was eluted and analyzed by Western blot analysis for AR and SRC-1.

Figure 6. Use of adenovector to evaluate new PSE promoter constructs. A. Schematic representation of recombinant Ad with enhancer constructs driving the luciferase reporter gene. Ad PSE-Bluc and AdPSE-BCluc were generated. The PSE-B construct is utilized for comparison because its regulatory region is very similar to CN706 which is currently being evaluated in clinical trials[22] (The PSA regulatory region of CN706 is -5322 to -3738 plus promotor -541 to +12. PSE-B construct is -5322 to -3744 plus promoter -541 to +12.). The luc expression cassette replaced the deleted E1 Ad sequence from 453 to 3323. **B.** Intracellular viral DNA analysis to ensure equivalent viral gene delivery. To accurately assess the specific promoter activity of the Ad PSE constructs, we needed to ensure equivalent infection. This was accomplished by first determining the infectious units of the viral stocks in plaque forming units/ml. We infected LNCaP cells equivalently with a ratio of 5 infectious virus per cell (MOI 5). The infected cells ($1x10^6$) were harvested and low molecular weight DNA, enriched for intracellular viral DNA were isolated. This DNA was analyzed by Southern Blot and probed with a digoxygenin labeled (Roche Co.) luciferase gene. **C.** The enhanced PSE-BC transcriptional activity and tissue specificity is retained in Ad. Different cell types are infected with AdPSE-Bluc or AdPSE-Bcluc equivalently at an MOI 5. LNCaP cells were first maintained for 24 hrs prior to infection in RPMI media with 10% charcoal stripped FBS. After infection, LNCaP cells are replaced with either this same media (-A) or with 1nM R1881 added (+A). All other cells are maintained in media containing 1nM R1881. R-11 is a renal carcinoma line.[30] The luciferase activity of AdPSE-BCluc infected LNCaP cells are 12 fold higher then equivalently infected with AdPSE-Bluc in the presence of androgen.

Figure 1A

NcoI BstEII BamHI

1 ***CCATGGTAAC C***GG***GGATCC***T CTAGAACTAG TGGATCTGC**A**

AREI (r) **AREI (f)**

41 **GAACAGCAAG TGCT**AGCTGA TCAGCT**AGCA CTTGCTGTTC**

AREI (f)

81 **T**GCAAGATCA GCT**AGCACTT GCTGTTCT**GC AAGCTCAGCT

AREI (f) **NcoI**

121 **AGCACTTGCT GTTCT**GCAAG ATCCCCCGGG C***CCATGG*** **157**

Figure 1B

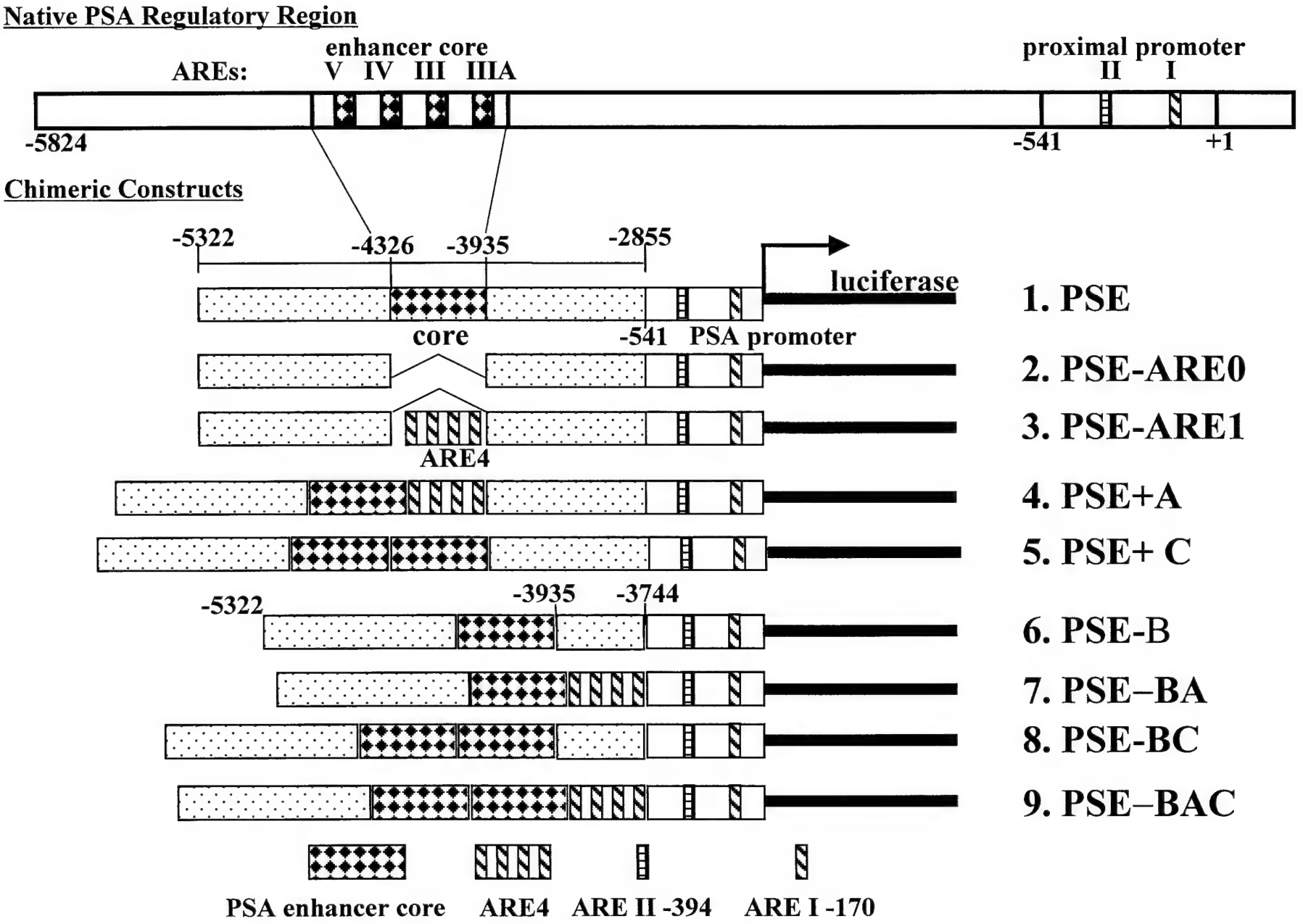

Native PSA Regulatory Region
enhancer core
AREs: V IV III IIIA
proximal promoter
II I
-5824
-541
+1
Chimeric Constructs
-5322
-4326
-3935
-2855
luciferase
core
-541 PSA promoter
ARE4
1. PSE
2. PSE-ARE0
3. PSE-ARE1
4. PSE+A
5. PSE+ C
-5322
-3935
-3744
6. PSE-B
7. PSE–BA
8. PSE-BC
9. PSE–BAC
PSA enhancer core
ARE4
ARE II -394
ARE I -170

Figure 2A

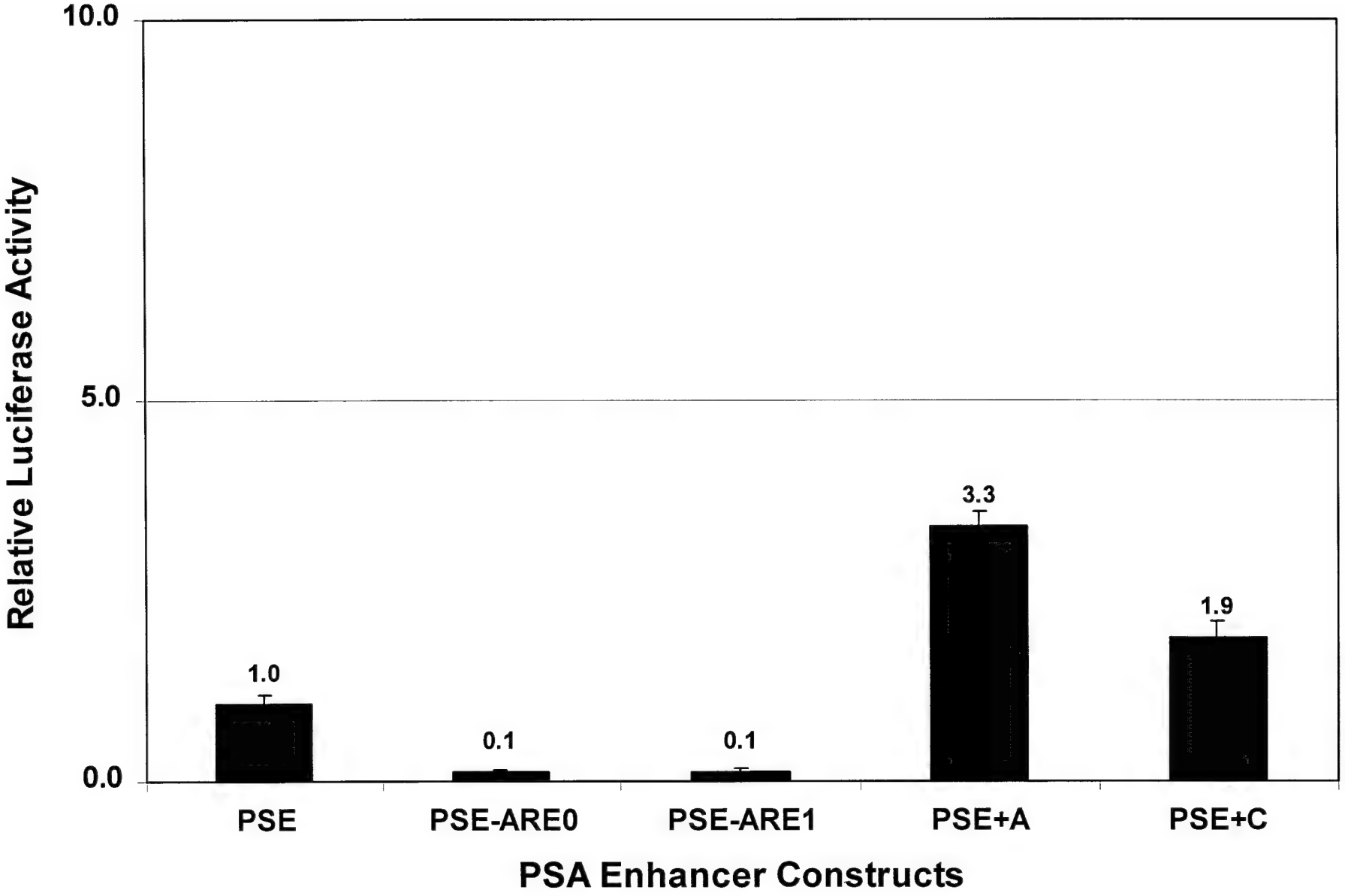

10.0
5.0
0.0
Relative Luciferase Activity
1.0
0.1
0.1
3.3
1.9
PSE
PSE-ARE0
PSE-ARE1
PSE+A
PSE+C
PSA Enhancer Constructs

Figure 2B

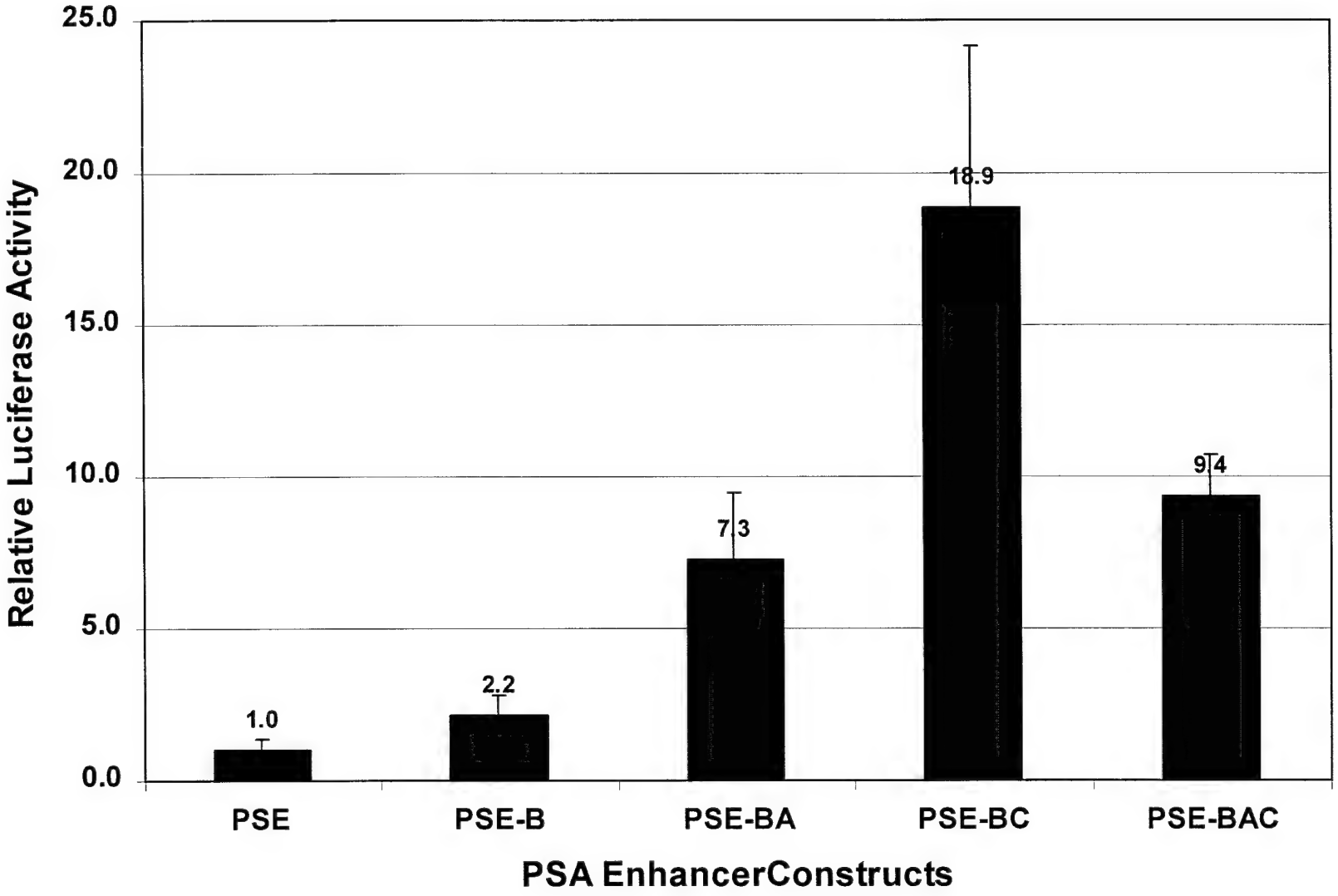

25.0
20.0
15.0
10.0
5.0
0.0
Relative Luciferase Activity
1.0
2.2
7.3
18.9
9.4
PSE
PSE-B
PSE-BA
PSE-BC
PSE-BAC
PSA EnhancerConstructs

Figure 3A

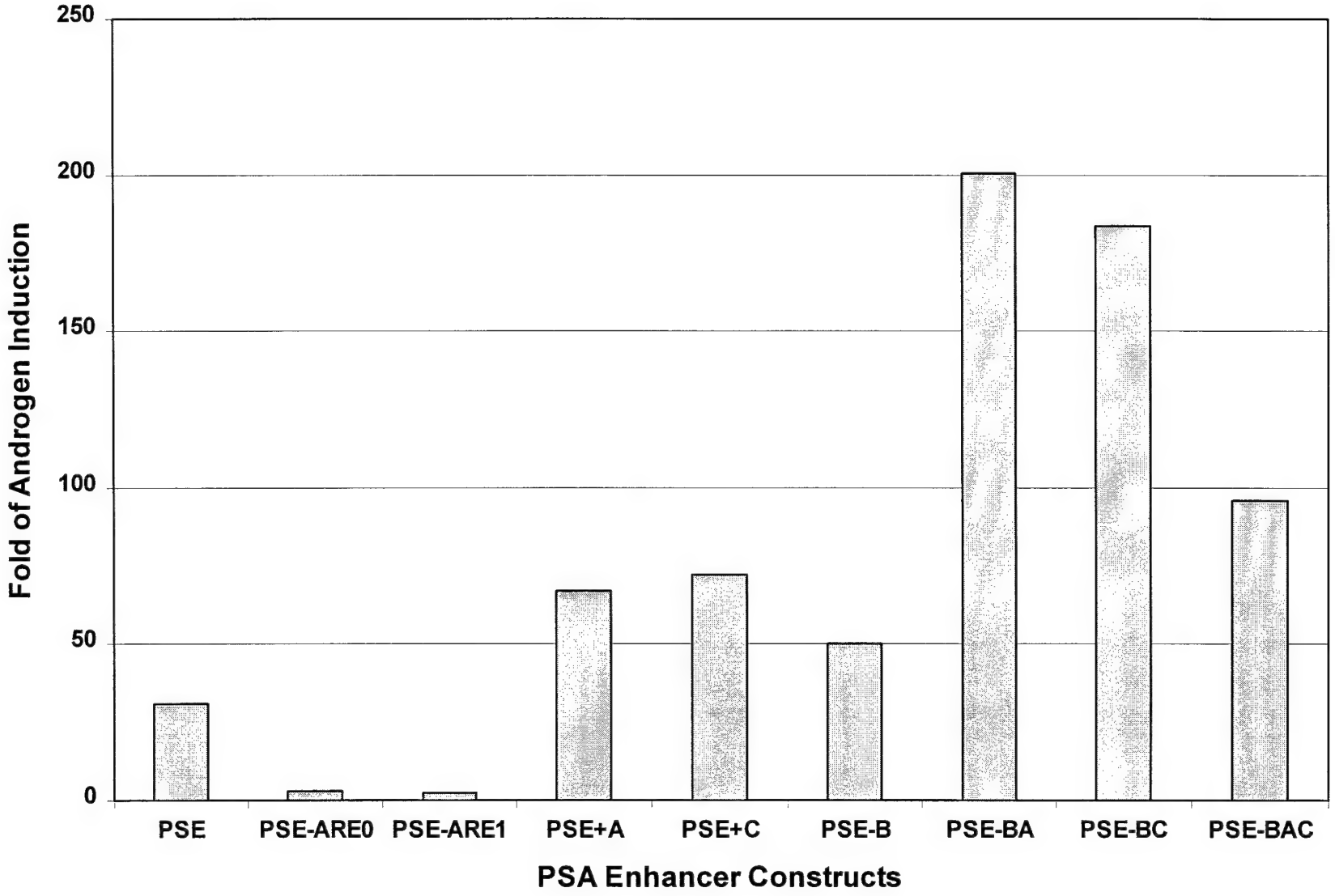

250
200
150
100
50
0
Fold of Androgen Induction
PSE
PSE-ARE0
PSE-ARE1
PSE+A
PSE+C
PSE-B
PSE-BA
PSE-BC
PSE-BAC
PSA Enhancer Constructs

Figure 3B

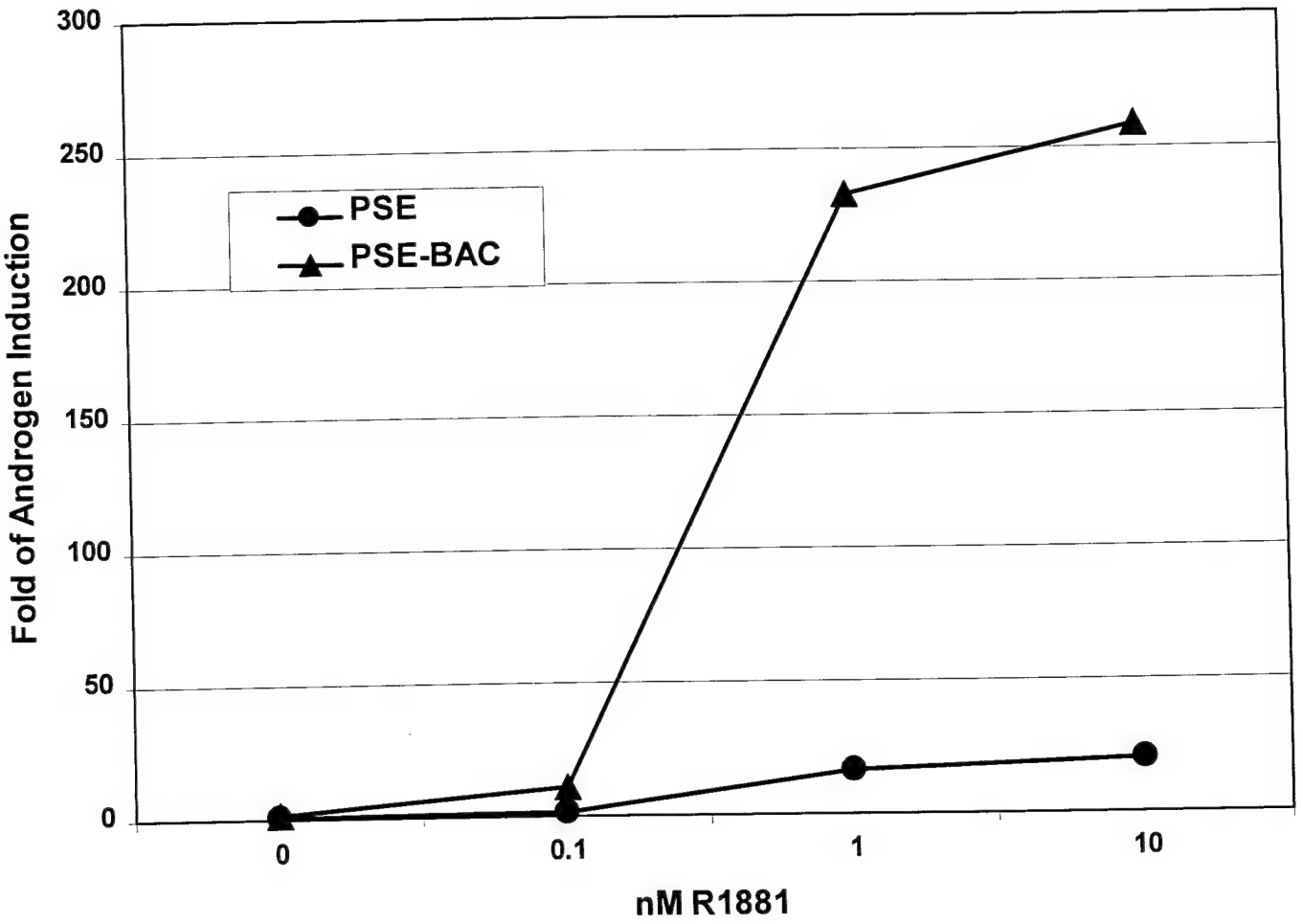
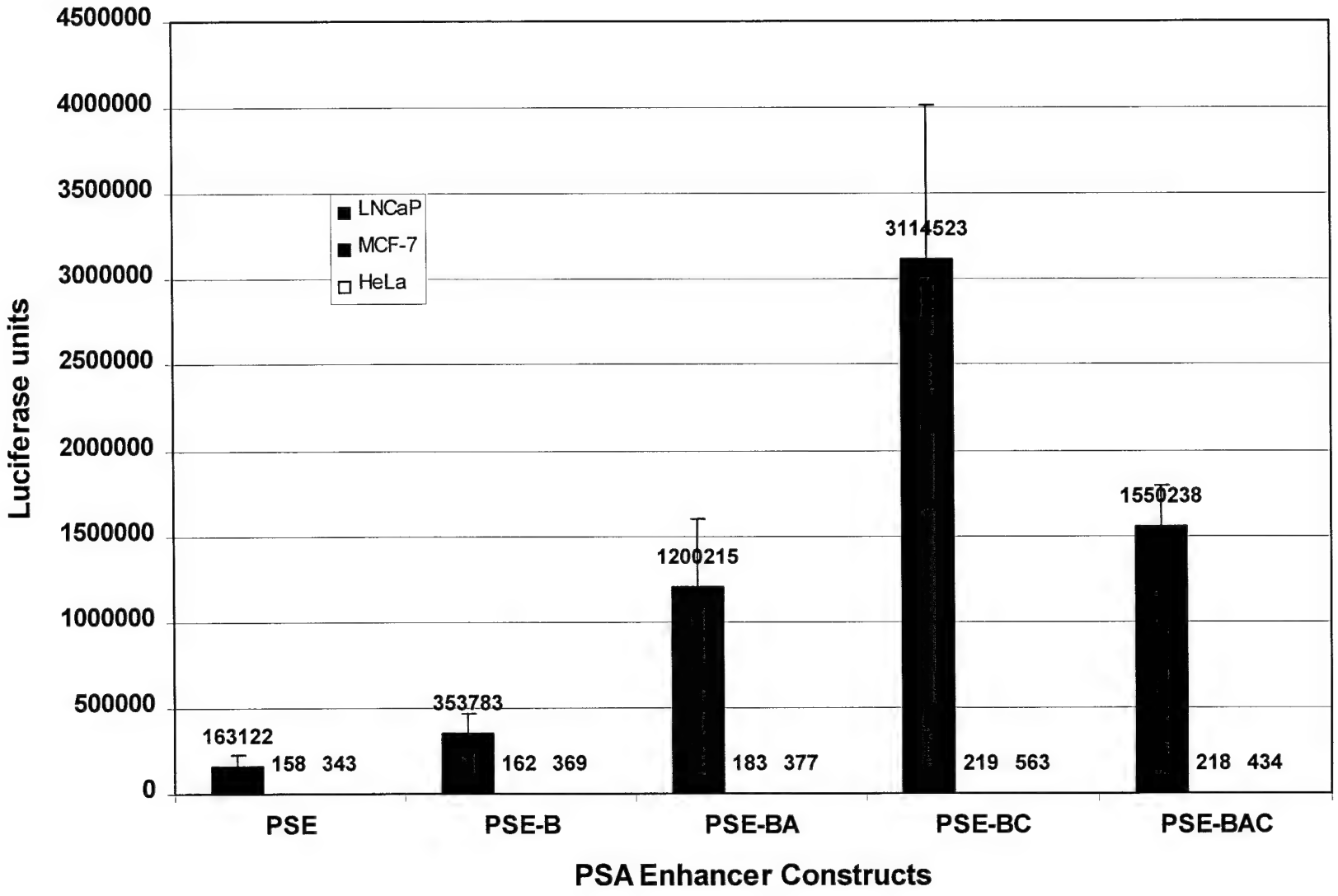

300
250
200
150
100
50
0
Fold of Androgen Induction
PSE
PSE-BAC
0
0.1
1
10
nM R1881

Figure 4A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4000000
3500000
3000000
2500000
2000000
1500000
1000000
500000
0
Luciferase units
LNCaP
MCF-7
HeLa
163122
158 343
353783
162 369
1200215
183 377
3114523
219 563
1550238
218 434
PSE
PSE-B
PSE-BA
PSE-BC
PSE-BAC
PSA Enhancer Constructs

Figure 4B

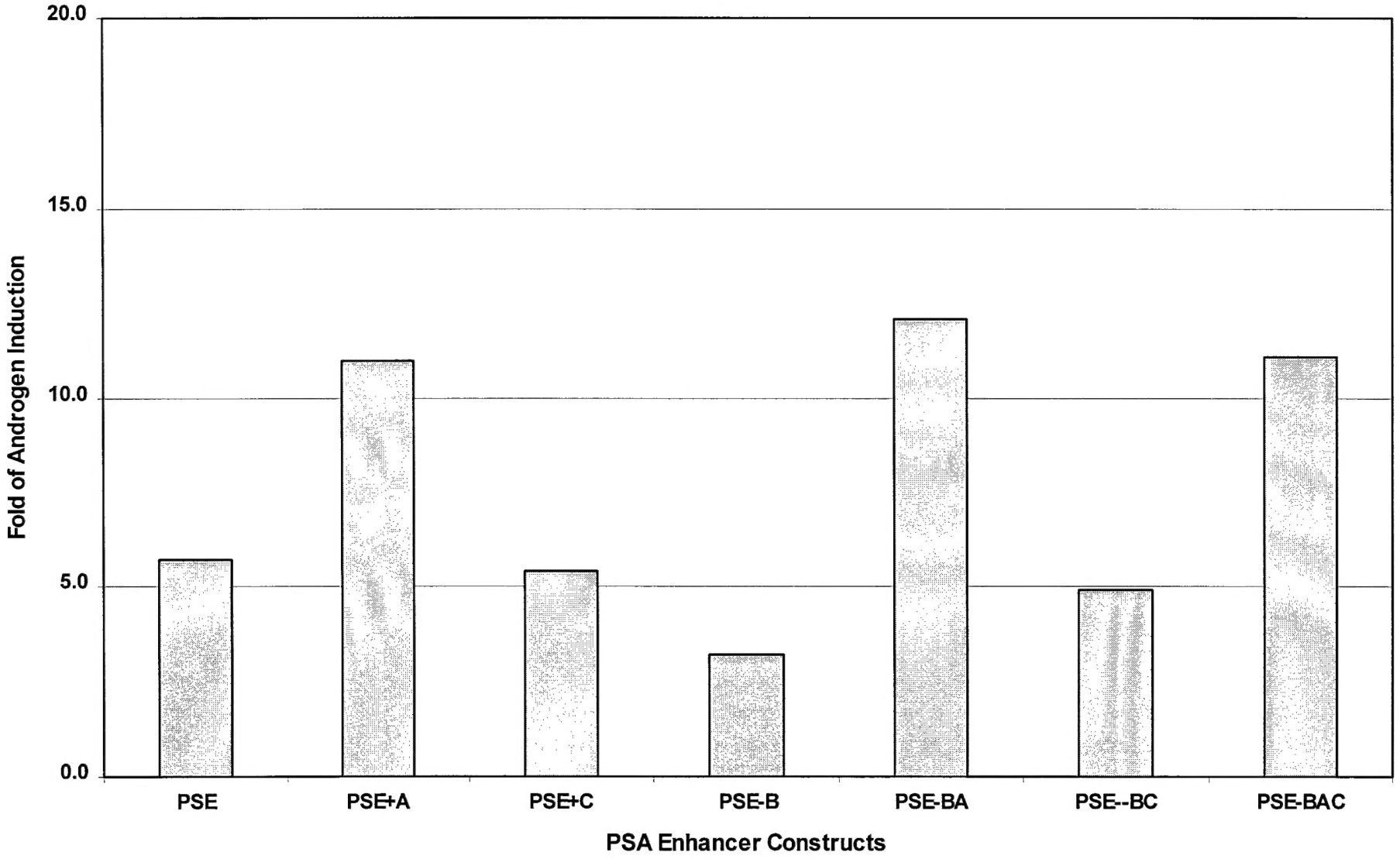

20.0
15.0
10.0
5.0
0.0
Fold of Androgen Induction
PSE
PSE+A
PSE+C
PSE-B
PSE-BA
PSE--BC
PSE-BAC
PSA Enhancer Constructs

Figure 5

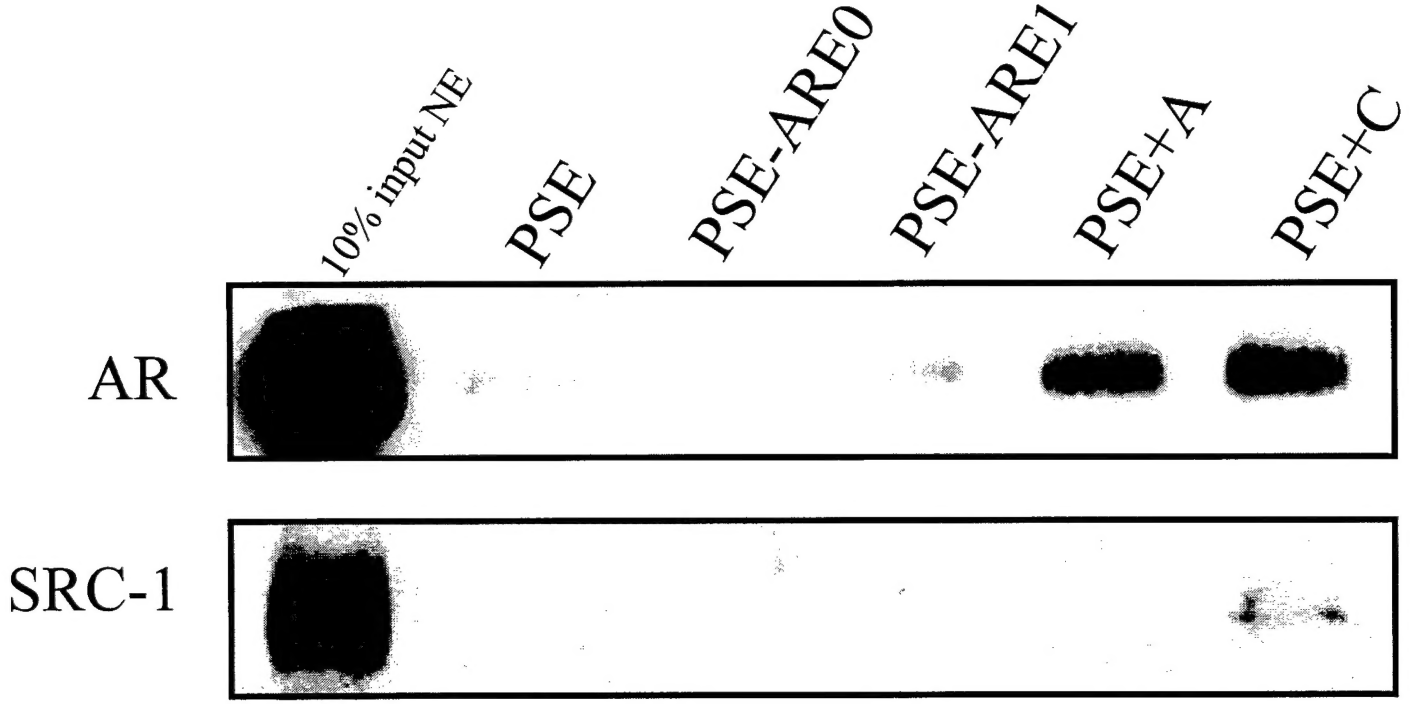


Figure 6A

Figure 6B

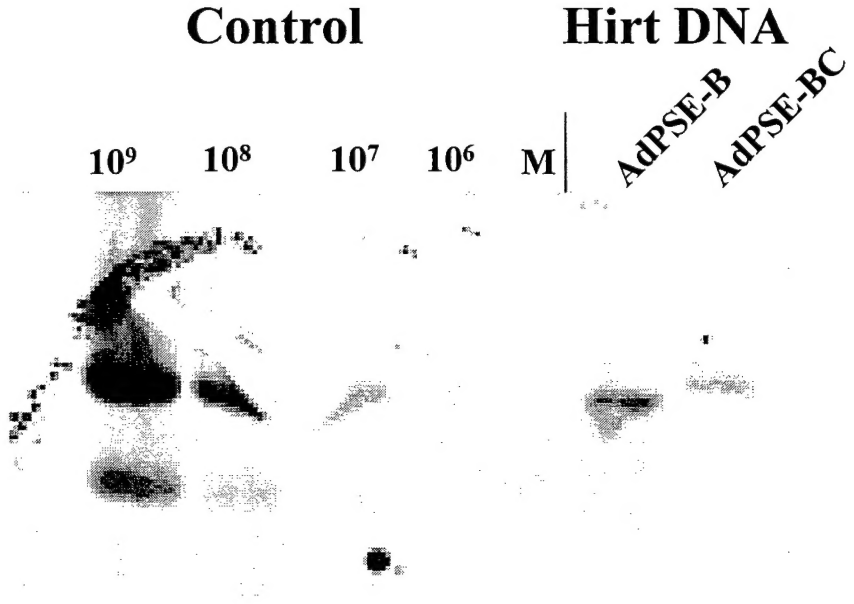
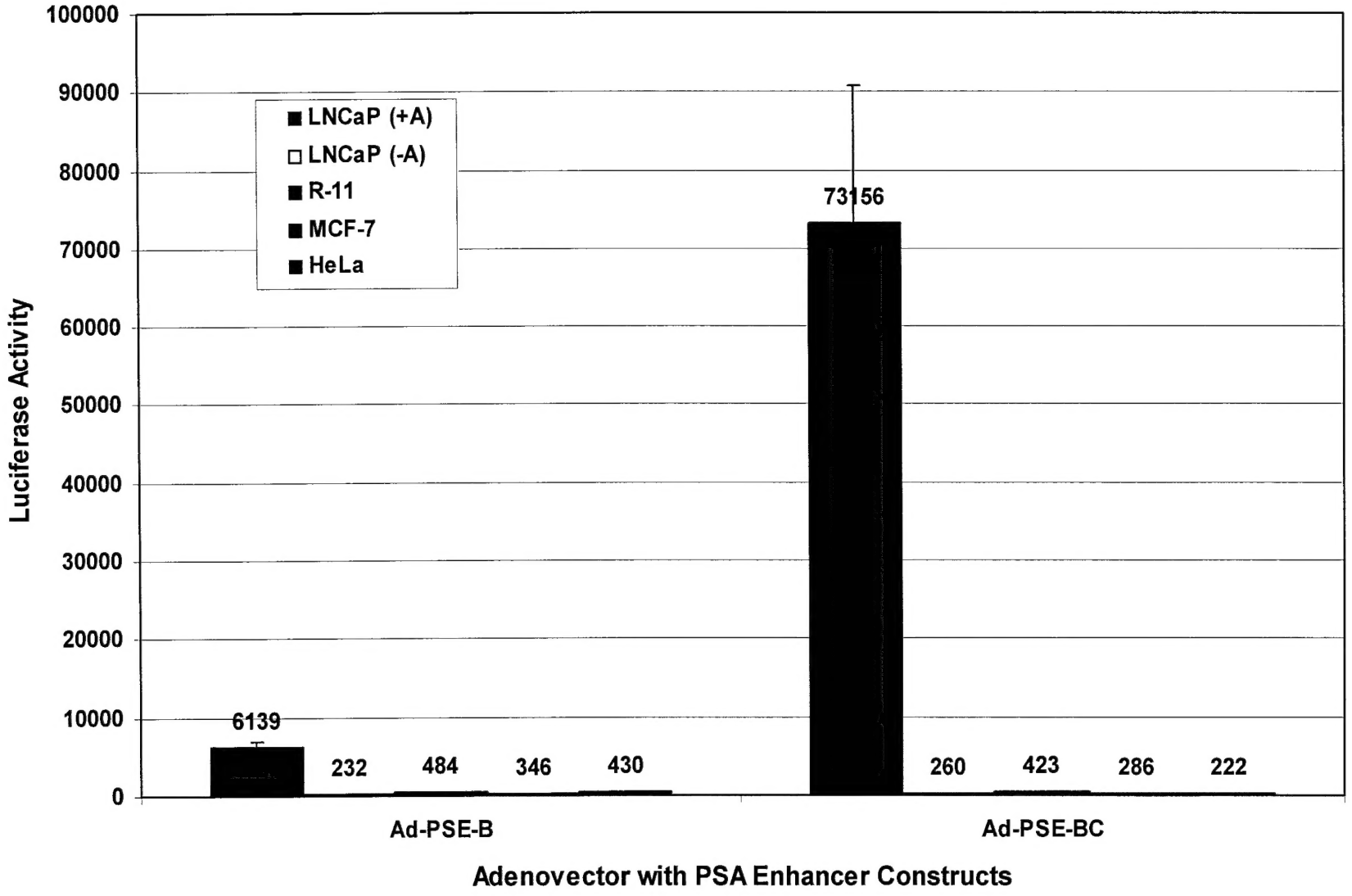


Figure 6C


100000
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
Luciferase Activity
LNCaP (+A)
LNCaP (-A)
R-11
MCF-7
HeLa
6139
232
484
346
430
73156
260
423
286
222
Ad-PSE-B
Ad-PSE-BC
Adenovector with PSA Enhancer Constructs